The influence of facultative endosymbionts on honeydew carbohydrate and amino acid composition of the black bean aphid *Aphis fabae*

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Abstract

The facultative endosymbionts *Hamiltonella defensa* and *Regiella insecticola* are commonly found in aphids. They are linked with various ecological benefits but generally occur at low prevalence, which indicates a possible harboring cost. Little is known about how the presence of facultative endosymbionts is reflected in honeydew composition. Honeydew is the key mediator of the aphids' mutualism with tending ants. The present study examines whether endosymbionts have an influence on aphid honeydew quality by comparing the amino acid and carbohydrate concentrations between infected and uninfected aphids. To this end, two genetic lines of the aphid *Aphis fabae* Scopoli were experimentally infected with different strains of *Hamiltonella* and *Regiella*. Infected aphids were shown to have reduced concentrations of amino acids in the honeydew compared to uninfected aphids. However, the presence of endosymbionts had no effect on the absolute amount of carbohydrates produced. Nevertheless, interclonal variation in honeydew composition between aphid genotypes was observed for both carbohydrate and amino acid production. These results imply that the nutritional value of honeydew depends on aphid genotype as well as on the presence of secondary bacterial endosymbionts, which suggests there is a physiological cost of harboring endosymbionts and which could also impact aphid attractiveness to tending ants.

Keywords: Aphids, Honeydew, Endosymbiosis, Mutualism, *Aphis fabae*, *Hamiltonella*, *Regiella*

Introduction

Insects have formed facultative or obligate relationships with multiple bacteria. This is called endosymbiosis when the bacteria reside inside the host (Buchner, 1965). Endosymbiotic bacteria have no free-living state and cannot propagate when taken out of their host (Hinde, 1971). In aphids, several endosymbioses have been well characterized, with almost all aphids harboring at least one symbiotic prokaryote, *Buchnera aphidicola* (Munson *et al.*, 1991). This symbiont plays a prominent role in nutrition by aiding in synthesis of nutrients that are lacking in the diet, mainly providing essential amino acids (Buchner, 1965; Douglas, 1996). Aside from the obligate mutualistic symbiosis between aphids and the intracellular bacteria *Buchnera*, which is commonly referred to as the primary symbiont (Buchner, 1965), aphids can also have several species of facultative secondary symbionts. The relationship of these secondary symbionts with their host can be parasitic, commensal or mutualistic, depending on the bacterial species and the environment (Douglas *et al.*, 2001; Zytynska & Weisser, 2015).

Beneficial ecological influences of the secondary endosymbionts *Hamiltonella defensa* and *Serratia symbiotica* have been shown to be related to heat tolerance (Chen *et al.*, 2000; Montllor *et al.*, 2002; Russell & Moran, 2006), whereas *Regiella insecticola* can increase fungal pathogen resistance (Scarborough *et al.*, 2005) and change host plant suitability (Tsuchida *et al.*, 2004). Furthermore, particular strains of all aforementioned endosymbionts can also increase parasitoid resistance (Oliver *et al.*, 2003; Vorburger *et al.*, 2009; Vorburger *et al.*, 2010; Asplen *et al.*, 2014). Recently, a combination of the aforementioned benefits was also discovered to be associated with carrying strains of an endosymbiotic bacterium known as X-type, which belongs to the Enterobacteriaceae (Heyworth & Ferrari, 2015). Parasitic influences are related to septicemia of tissues leading to death of the host (Costechareyre *et al.*, 2012) and negative effects on growth, fecundity and longevity of the host (Fukatsu *et al.*, 2001; Simon *et al.*, 2011; Vorburger & Gouskov, 2011). From the experiments mentioned above, it is clear that the impact of the endosymbionts, and whether they are beneficial or detrimental, is highly species and genotype dependent (Koga *et al.*, 2007; Asplen *et al.*, 2014;

66 Vorburger, 2014) and sometimes even reliant on symbiont by symbiont interaction effects (Oliver *et*
67 *al.*, 2006; Tsuchida *et al.*, 2014). Secondary symbionts can also play a role in aphid nutrition. The
68 symbiont *Serratia*, for example, has been shown to synthesize several essential amino acids, thereby
69 successfully replacing the function of *Buchnera* (Douglas & Prosser, 1992; Koga *et al.*, 2003), and it
70 has even evolved into a co-obligate symbiont in aphids of the genus *Cinara* (Lamelas *et al.*, 2011).
71 However, symbionts *Regiella* and *Hamiltonella* have lost all essential amino acid biosynthetic
72 pathways except for those relating to the production of two essential amino acids, threonine and
73 lysine (Degnan *et al.*, 2009; Degnan *et al.*, 2010).

74 Aphids are very successful and often considered as pest species because of their impact on
75 agricultural crops, causing damage by direct feeding and by transmission of plant diseases. Aphids
76 are plant-sucking insects and can become very numerous thanks to their predominantly asexual
77 mode of reproduction. The diet of the aphids consists of the phloem sap of plants, a sugar-rich but
78 nitrogen and amino acid-poor fluid. As a consequence of this diet aphids excrete honeydew, a waste
79 product that contains large amounts of both simple and complex carbohydrates and also a small
80 amount of amino acids and vitamins (Mittler, 1958). Osmoregulation is widely accepted as the main
81 reason for honeydew and complex carbohydrate production (Downing, 1978; Fisher *et al.*, 1984;
82 Wilkinson *et al.*, 1997; Douglas, 2003), but honeydew composition can be influenced by several
83 factors. Honeydew carbohydrate composition is variable between aphid species (Volkl *et al.*, 1999),
84 aphid genotypes (Vantaux *et al.*, 2011; Katayama *et al.*, 2013), aphid instars (Fischer *et al.*, 2002),
85 host plants (Volkl *et al.*, 1999; Fischer & Shingleton, 2001; Fischer *et al.*, 2005), seasons (Wool *et al.*,
86 2006) and can be influenced by ant-tending (Fischer & Shingleton, 2001; Yao & Akimoto, 2002).
87 Amino acid composition is variable between aphid species (Sandström & Moran, 2001; Woodring *et*
88 *al.*, 2004), aphid instars and the presence or absence of primary endosymbiont *Buchnera* (Sasaki *et*
89 *al.*, 1990) and can increase in response to ant-tending (Yao & Akimoto, 2002). However, there is no
90 indication of a different amino acid composition between host plants (Sandstrom & Pettersson,
91 1994) or in function of seasonal differences in phloem sap composition (Yao & Akimoto, 2002).

Honeydew is a key factor that drives the mutualism between ants and aphids. It is a high nutrition food source for ants and in return the ants protect aphids against natural enemies (Nixon, 1951; Banks, 1962; Buckley, 1987). The mutualistic interaction between ants and aphids could also exert a selective pressure on the composition of honeydew, to maximize the attractiveness and nutritional value for the ants (Volkl *et al.*, 1999) as the aphids benefit from the visits of ants (Hölldobler & Wilson, 1990; Flatt & Weisser, 2000).

This study investigates the role of the facultative secondary endosymbionts *Hamiltonella* and *Regiella* on the honeydew composition of *Aphis fabae* Scopoli, and thereby evaluates if harboring endosymbionts could influence ant-aphid mutualisms. Presence of endosymbionts would be expected to affect honeydew composition if the endosymbionts compete for (i) host niche with *Buchnera*, thereby resulting in reduced amino acid production by *Buchnera* (Koga *et al.*, 2003; Sakurai *et al.*, 2005) and/or (ii) essential amino acids and carbohydrates (Chandler *et al.*, 2008). Our results demonstrate that presence of the secondary endosymbionts decreased total amino acid concentrations in the honeydew, but that they had no effect on carbohydrate concentrations in the honeydew. We suggest that the decrease in amino acid concentration could indicate a physiological or ecological cost of harboring the secondary endosymbionts.

Material and methods

Study organism

The studied black bean aphid (*Aphis fabae*) is a common aphid species in the northern hemisphere. It is a polyphagous species and has a wide range of secondary host plants ranging from cultivated to wild plants (Stroyan, 1984). Based on those secondary host plants it is possible to distinguish four different subspecies of *A. fabae*. In this study, we focused on *A. fabae fabae*, which has broad bean (*Vicia faba*) as the main summer host plant. The winter host plants, mainly the European spindle tree

(*Euonymus europaeus*), are used in autumn for winter survival of eggs deposited after sexual reproduction. In spring the eggs hatch and viviparous, parthenogenetic females start reproducing, after which offspring migrates to the secondary host plants where they reproduce asexually for the remainder of the spring and summer. The honeydew of *A. fabae* is highly dependent on genotype, with especially the production of the trisaccharide melezitose being highly variably across aphid genotypes (Vantaux *et al.*, 2011). The studied aphids belong to two clones collected in Switzerland on *Chenopodium album*, hereafter referred to as clone A and B. Clone A can be categorized as a low melezitose clone with melezitose levels <1%, and clone B as a high melezitose clone with melezitose levels >20% in honeydew samples.

A PCR screening showed that both clones were clear of any of the known secondary symbionts (Vorburger *et al.*, 2009). Subsequently, they were experimentally infected with one of each of two strains of *Regiella* (R49 and R5.15) or one of each of four strains of *Hamiltonella* (H30, H323, H76, HAF6). This generated 13 different lines, as infection of R5.15 only succeeded in one of the aphid genotypes. Hereafter, the different infected lines are referred to respectively as R1, R2, H1, H2, H3 and H4. Infection occurred via microinjection, as described in Vorburger *et al.* (2010). The clonal lines were propagated asexually on *Vicia faba* plants (seeds from Somers Seeds NV, Mechelen, Belgium) under controlled laboratory conditions (LD 16:8 h photocycle, 20 ± 1 °C and 65-70% humidity).

Collection of honeydew

Vicia faba plants were grown from seeds in universal garden soil. Two weeks after sowing, the plants were inoculated with aphids to collect honeydew as previously described in Vantaux *et al*. (2011). Ten aphids (adults or fourth instar) were transferred to an apical leaf enclosed in a plastic box (50x25x30 mm). After 24h the numbers of aphids that survived were counted to take into account how many aphids had successfully settled on the plant and therefore had contributed to the production of the honeydew. This survival measurement does not distinguish between a settlement

cost and survival costs related to the presence of endosymbionts. The box and leaf were rinsed with 400 µL of a 0.02% sodium azide solution. The obtained mixture was boiled for 5 min, dried out overnight in an oven at 60 °C, re-dissolved in 200µL of 0.02% sodium azide solution and stored at -20 °C until further analysis. For every aphid line 5 to 7 samples were obtained following this protocol.

Carbohydrate and amino acid analysis

Before carbohydrate analysis, all samples were passed through a mixed-bed Dowex column to obtain a neutral fraction (Van den Ende *et al.*, 1996). This was done by allowing 50 µl of sample solution followed by 1.2 mL of distilled water to pass through the column. The samples were then centrifuged for 5 min and stored at -20 °C until they could be chemically analysed. To analyse the amino acids, each sample solution was diluted 5 times before the chemical analyses.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex ICS 3000, Sunnyvale, California) was used to analyse the carbohydrates and amino acids present in all honeydew samples (see protocol described in Vantaux *et al.* (2011) and Gijbels *et al.* (2014). The molar concentrations of the different carbohydrates and amino acids in each analysed sample were estimated by comparing the area under the chromatogram peaks with standards using the software package Chromeleon (Dionex, Sunnyvale, California). Overall we could detect 8 different carbohydrates and all 20 primary amino acids in the honeydew samples. Due to column interference only 17 amino acids were used in the final analyses. The missing amino acids had only minor contributions to the total amino acid concentration in the honeydew samples.

Statistical analysis

To analyse the absolute concentrations of all compounds found in the honeydew we used linear mixed models (Bates *et al.*, 2014) with a Gaussian distribution on log transformed data to fulfill the assumptions. The full model consisted of a random intercept model with aphid genotype, symbiont species and their interaction as fixed factors, the number of aphids coded as a continuous covariate and symbiont strain nested within the species and replicate coded as random intercepts, to take into account repeated measurement of the same aphid genotypes (Schank & Koehnle, 2009). A backward stepwise model simplification based on the Akaike information criterion (AIC) was used to arrive at a minimum adequate model considering the structure of the experimental setup. This resulted in omitting the interaction term for all compounds. All results are displayed as least square means and 95% CI. Species differences were corrected for multiple comparisons with a Tukey's post hoc test. All statistical analyses were performed in R 3.2.0 (R core team, 2015).

Results

Amino acids

A general pattern of reduced total amino acid concentration was observed in the lines infected with secondary endosymbionts. A decrease of 51 % was detected for *Hamiltonella* (347.72 CI [280.14-431.6]; P-value=0.008) and a 47 % decrease for *Regiella* (373.05 CI [261.13-532.93]; P-value=0,04) compared to the uninfected lines (706.95 CI [461.73-1082.41]) (Fig. 1a). For 9 of the 17 individual amino acids this trend was significant for at least one endosymbiont species (Fig. 2).

The major amino acid compounds (>10% of total amino acid concentration) were arginine, asparagine and glycine for both aphid genotypes. Minor amino acid compounds (<1% of total amino acid concentration) were glutamine, ornithine and methionine, with methionine absent from all honeydew samples. For 11 of the 17 individual amino acids there were significant differences in the concentration between the aphid genotypes (Fig. 3) with a general trend of lower concentrations in

clone B, resulting in a 36 % lower total concentration of amino acid in the honeydew compared to clone A (clone A: 506.13 CI [355.91-621.97]; clone B: 324.40 CI [240.27-391.21]; P-value: 0.01) (Fig. 1b).

Carbohydrates

The two aphid clones showed significant differences in concentration of several carbohydrates. Clone A showed increased fructose (P-value= 0.03), maltose (P-value= 0.005) and maltotriose (P-value= 0.02) production but a decreased melezitose (P-value <0.001) production compared to clone B. The total concentration of carbohydrates did not show significant differences between the two clones (Fig. 4). The monosaccharide fructose was the most abundant sugar followed by glucose for both aphid clones and as already known the trisaccharide melezitose was only detected in clone B. However, the endosymbionts showed no significant influence on the concentration of the carbohydrates found in the honeydew (P-value=0.54).

Discussion

Little is known about how the presence of facultative endosymbionts *Hamiltonella* and *Regiella* influences the composition of aphid honeydew. The present results clearly demonstrate that secondary endosymbionts have an impact on honeydew quality, thus suggesting that there is a significant cost to harbor them. Indeed, the amount of amino acids found in the honeydew of infected aphids was consistently reduced for both *Hamiltonella* and *Regiella* infected aphids compared to uninfected aphids, while the total amount of carbohydrates was not affected. A harboring cost is also consistent with the fact that secondary endosymbionts occur at moderate or low frequencies despite conferring specific benefits (Oliver *et al.*, 2006; Vorburger & Gouskov, 2011; Zytynska & Weisser, 2015). Presence of endosymbionts would be expected to be costly if they compete for host niche and/or limited resources with *Buchnera,* resulting in reduced *Buchnera*

densities and amino acid production. Both primary and secondary endosymbionts are hosted in special cells and declining densities of *Buchnera* with aphid age have been observed when a coinfection occurred with *Serratia,* whereas *Serratia* densities increased until a plateau was reached (Koga *et al.*, 2003). Similarly, co-infection with an endosymbiont of the genus *Rickettsia,* another common facultative endosymbiont in aphids, resulted in lower densities of *Buchnera* (Sakurai *et al.*, 2005). An additional cost can arise if secondary endosymbionts compete for essential amino acids. The infection of *A. fabae* with *Hamiltonella* and *Regiella* showed a diet-dependent impact on aphid fitness with a reduced performance when reared on diets with lower amino acid concentrations (Chandler *et al.*, 2008). Further research, however, would be required to determine which of these factors best explain the reduced amino acid secretion in the honeydew of *A. fabae*.

Depending on aphid species, different essential amino acids may be absent from the honeydew (Woodring *et al.*, 2004). In our experiment, for example, the essential amino acid methionine was absent from all honeydew samples. This is a common pattern, as methionine has also been shown to be absent from the honeydew of other aphid species feeding on wheat (Sandström & Moran, 2001), broad bean plants (Sasaki *et al.*, 1990) and tansy (Woodring *et al.*, 2004). The absence of essential amino acids from honeydew suggest the presence of resource limitations, and resource limitation combined with competition from secondary endosymbionts has earlier been shown to result in significant fitness costs in *A. fabae* (Chandler *et al.* 2008).

Besides the intraspecific variation in melezitose production, this study reveals an aphid genotype-dependant variation in amino acid concentration as well. More genotypes should be studied to know if the detected decrease of amino acid concentration found in the honeydew of the low melezitose genotype is linked to the lack of melezitose production or to aphid genotype-dependent population sizes of the primary endosymbiont *Buchnera* (Zhang et al., 2016). Alternatively, the significant difference in amino acid concentration in the honeydew could indicate a difference in resilience to the presence of secondary endosymbionts (Scarborough *et al.*, 2005; Russell & Moran, 2006; Łukasik *et al.*, 2013) .

Finally, melezitose concentrations also showed aphid genotype-related variation, as had previously been observed by Vantaux *et al.* (2011). The lack of melezitose production in clone A, however, was completely compensated for by increased production of the other carbohydrates, as there was no notable difference in total amount of carbohydrates found in the honeydew of the two aphid clones. Vantaux *et al.* (2011) compared the honeydew of several low- and high-melezitose *A. fabae* clones and found interclonal differences in glucose, melezitose and total carbohydrate production but no general trends to explain the lack of melezitose in low-melezitose clones. In our study, clone A seemed to compensate the lack of melezitose production mainly by excreting the monosaccharide fructose. The presence of oligosaccharides, e.g. melezitose, in the honeydew of aphids are generally seen as a mechanism to deal with the osmotic stress related to the ingestion of large amounts of phloem sap (Downing, 1978; Fisher *et al.*, 1984; Wilkinson *et al.*, 1997; Douglas, 2003). However, from our results, it appears that clone A must have evolved a different strategy to deal with osmotic pressure without affecting honeydew production or fitness (Vantaux *et al.*, 2011; Vantaux *et al.*, 2015). In the future, this intraspecific variation may be exploited as a model for further studies into the regulation of gut osmolarity in aphids.

Previous results have shown that melezitose is also an important carbohydrate in the ant-aphid mutualism (Kiss, 1981; Volkl *et al.*, 1999; Woodring *et al.*, 2004; Detrain *et al.*, 2010). Higher melezitose concentrations in honeydew, for example, have been shown to increase ant attendance (Volkl *et al.*, 1999; Woodring *et al.*, 2004). The ant – aphid interaction is mainly based on honeydew as a carbohydrate source and even though for many honeydew collecting ant species this source seems to fulfill most nutritional demands (Alsina *et al.*, 1988; Fiedler *et al.*, 2007; Domisch *et al.*, 2009), predation of aphids is also observed (Sakata, 1994; Offenberg, 2001; Billick *et al.*, 2007). Besides variability in carbohydrates, amino acid variability is proposed to play a role in ant preference (Blüthgen & Fiedler, 2004). The nutritional requirements of ants, especially the protein demands, can have seasonal fluctuations that go along with brood production and increased predation rate on aphids (Abril *et al.*, 2007). In general, ants prefer a carbohydrate solution with amino acids over a

pure carbohydrate solution (Blüthgen & Fiedler, 2004) and ant-tended aphid species have higher amino acid concentrations (Lanza *et al.*, 1993; Woodring *et al.*, 2004). Some ant species have also been shown to prefer amino acid-rich food sources (Lanza *et al.*, 1993; Lee Cassill & Tschinkel, 1999). In addition, aphids appear to be able to maintain a constant amino acid concentration across different seasons (Yao & Akimoto, 2002) and when reared on different host plants (Sandstrom & Pettersson, 1994), and the drepanosiphid aphid (*Tuberculatus quercicola*) produced more amino acid-rich honeydew when ant tended (Yao & Akimoto, 2002). Overall, these results indicates that the ant-aphid mutualism exerts positive selection for an increased nutritional value of honeydew. Consequently, the presence of secondary endosymbionts and concomitant changes in the nutritional value of the honeydew would also be expected to affect the ant-aphid mutualism and ant attractiveness. Earlier, volatile compounds from bacterial origin have also been shown to affect the ant attractiveness of aphids (Leroy *et al.*, 2011; Fischer *et al.*, 2015). Finally, the presence of secondary endosymbionts could induce a higher predation rate, to compensate the reduced amino acid concentration in the honeydew and satisfy the nutritional requirements of the tending ants. In the future, these ideas would be interesting to test further in controlled laboratory trials.

Overall, our study shows that aphid honeydew composition is affected not just by aphid genotype but also by the presence or absence of secondary endosymbionts. These results suggest that harboring secondary endosymbionts carries significant fitness costs. Future studies could be aimed at testing whether the altered nutritional value of the honeydew also impacts the ant-aphid mutualism.

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481 **Figure Legends**

482 **Fig. 1.** Total amino acid concentration found in honeydew of *Aphis fabae* for (a) the uninfected
483 (none) and infected lines with *Regiella insecticola* (R) and *Hamiltonella defense* (H) and (b) the two
484 aphid genotypes. Significant differences after post-hoc test (Tukey) are indicated with * for $P \leq 0.05$.

485 **Fig. 2.** Mean concentration of the minor (<1%), intermediate (<10%), and major (>10%) amino acid
486 components found in honeydew of *Aphis fabae* for the uninfected (none) and infected lines with
487 *Regiella insecticola* and *Hamiltonella defensa* (% of total amino acid concentration). Significant
488 differences after post-hoc correction (Tukey) are indicated with * for $P \leq 0.05$, ** $P \leq 0.01$ and with
489 *** for $P \leq 0.001$.

490 **Fig. 3.** Mean concentration of the minor (<1%), intermediate (<10%), and major (>10%) amino acid
491 components found in honeydew of *Aphis fabae* for the two aphid genotypes (% of total amino acid
492 concentration). Significant differences between the genotypes for individual components are
493 indicated with * for $P \leq 0.05$, ** $P \leq 0.01$ and with *** for $P \leq 0.001$.

494 **Fig. 4.** Mean concentration of the (a) minor (<5%) and (b) major (>5%) compounds and the total
495 carbohydrate concentration found in honeydew of *Aphis fabae* for the two aphid genotypes (% of
496 total carbohydrate concentration). Significant differences between the genotypes for individual
497 compounds is indicated with * for $P \leq 0.05$, ** $P \leq 0.01$ and with *** for $P \leq 0.001$.

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